

Automated Detection of Toxigenic *Clostridium difficile* in Clinical Samples: Isothermal *tcdB* Amplification Coupled to Array-Based Detection

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Clostridium difficile can carry a genetically variable pathogenicity locus (PaLoc), which encodes clostridial toxins A and B. In hospitals and in the community at large, this organism is increasingly identified as a pathogen. To develop a diagnostic test that combines the strengths of immunoassays (cost) and DNA amplification assays (sensitivity/specificity), we targeted a genetically stable PaLoc region, amplifying *tcdB* sequences and detecting them by hybridization capture. The assay employs a hot-start isothermal method coupled to a multiplexed chip-based readout, creating a manual assay that detects toxigenic *C. difficile* with high sensitivity and specificity within 1 h. Assay automation on an electromechanical instrument produced an analytical sensitivity of 10 CFU (95% probability of detection) of *C. difficile* in fecal samples, along with discrimination against other enteric bacteria. To verify automated assay function, 130 patient samples were tested: 31/32 positive samples (97% sensitive; 95% confidence interval [CI], 82 to 99%) and 98/98 negative samples (100% specific; 95% CI, 95 to 100%) were scored correctly. Large-scale clinical studies are now planned to determine clinical sensitivity and specificity.

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacterium. Infection by toxin-producing *C. difficile* causes a spectrum of disease from mild diarrhea to fulminant pseudomembranous colitis (4, 27). Although *C. difficile* is apparently an ancient species, having emerged more than 1 million years ago (18), it has been recognized as a human pathogen for only 3 decades (5), with dramatic increases in both hospital- and community-acquired infections in the past decade (6). More than likely, alterations in human behavior such as increased antibiotic use and more frequent hospitalizations have combined with adaptations within the mobile *C. difficile* genome to generate this pathogenic emergence. Estimated hospital costs per infected patient ranged from \$2,500 to \$7,000 in the mid-2000s (14, 30). Prior antibiotic treatment increases infection risk, presumably because loss of normal flora enables *C. difficile* to propagate in a less competitive environment (3).

Toxigenic *C. difficile* strains contain a pathogenicity locus (PaLoc) that harbors genes encoding the large clostridial toxins A and B (26). Though the individual roles of *tcdA* and *tcdB* genes remain a subject of investigation (2), for diagnosis of toxigenic status a stable genetic marker within the PaLoc is the singular requirement. *C. difficile* maintains a variable/plastic genome (29), and the PaLoc flanking sequences show evidence of insertion by a mobile genetic element (9). Various alterations that leave remnants or mutated forms of both *tcdA* (16, 25) and the regulatory gene *tcdC* (8) are described in toxigenic *C. difficile* isolates, and previously unknown *tcdA* deletions are still being reported (15). Such variability underscores the diagnostic requirement for a stable toxicity marker(s) within the PaLoc.

Diagnostic testing for toxigenic *C. difficile* has been traditionally accomplished by time-consuming culture methods and by immunoassays, which are faster but in general do not have sufficient sensitivity. Immunoassays that detect the glutamate dehydrogenase (GDH) antigen display high sensitivity but poor spec-

ificity for *C. difficile*. Further, the GDH assays do not determine toxigenic status. This led some laboratories to adopt two-step algorithms in which samples that test GDH positive are further tested to determine whether the identified *C. difficile* is toxigenic (17). In comparison to such two-step algorithms, molecular tests alone have increased sensitivity/specificity (96%/97%) (21) but are more costly. To combine the advantages of molecular-only and two-step algorithms, we have developed a cost-effective molecular test that couples isothermal DNA amplification to visual chip readout.

MATERIALS AND METHODS

Primers and probes. To develop the amplification primers, we sought an evolutionarily conserved genetic region that is common to all toxigenic *C. difficile* isolates. Twenty-two sequences, including the PaLoc from the five major evolutionary *C. difficile* branches (18) and the related *Clostridium sordellii* toxin gene, *tcsL* (GenBank accession number X82638), were downloaded from GenBank and aligned (CLC Sequence Viewer; CLC Bio, Aarhus, Denmark). Several oligonucleotide primer pairs were designed using previously identified parameters for helicase-dependent amplification ([HDA] IsoAmp II Universal HDA Kit package insert; BioHelix, Beverly, MA). Primer candidates were screened *in silico* for potential dimer formation (32) as well as for hairpin and self-dimer formation using DNA folding algorithms (35) and an algorithm (Integrated DNA Technologies, Coralville, IA) that employs nearest-neighbor thermodynamic parameters (28). BLASTN (19) analysis was performed for primer

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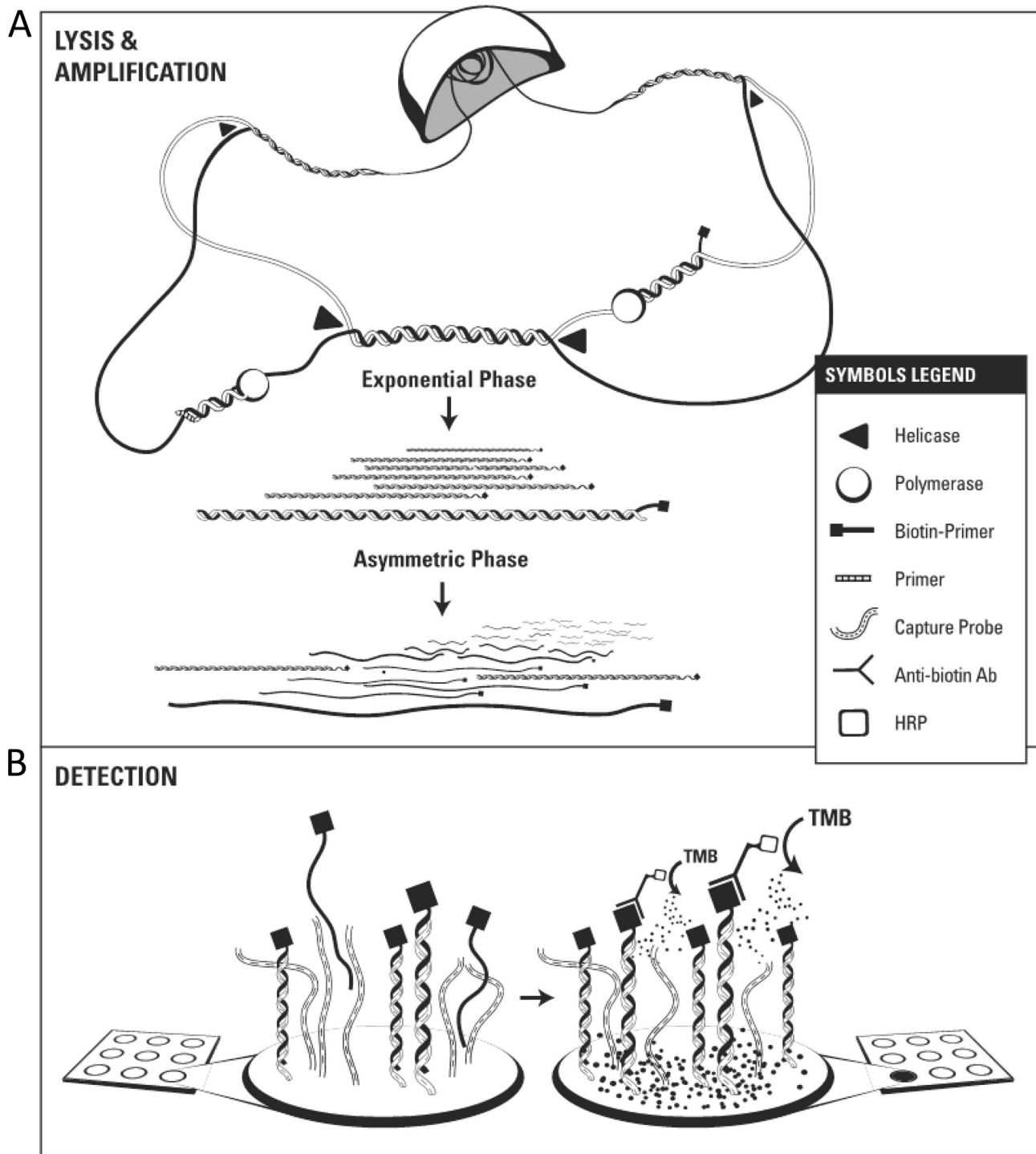
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and probes to determine potential cross-reactivity to nontarget sequences.

Primers were screened in HDA reactions (1) for rapid amplification and minimal artifact formation, as judged by product melting point (T_m) and electrophoretic mobility relative to size standards. To develop an internal control for DNA extraction and amplification, a primer set and capture probe were synthesized that amplify and detect the thermonuclease (*nuc*) gene of *Staphylococcus aureus*. Primer sequences for *tcdB* were 5'-TAC AGATGAATATATTGCAGCAACTG[G]TTCA-3' and 5'-TTGAGCTGT ATCAGGATCAAAATAATACTC[C]TCAC (where bracketed letters represent ribonucleotide positions) (targeting GenBank accession number NC_009089.1; *tcdB* amino acids 2337 to 2362); for *nuc* the primer sequences were 5'-TGGTAGAAATGCAAAGAAATTGAAGTC[G]AGTT and 5'-TCCATCAGCATAAATATACGCTAAGCCA[C]GTCC (targeting GenBank accession number CP000730; amino acids 143 to 175). DNA capture probes were designed using MeltCalc (34), which uses nearest-neighbor calculations to optimize discrimination between sequences. To distinguish amplified *tcdB* from *tcsL*, a homologous toxin gene from *Clostridium sordellii*, a *tcdB*-specific probe was designed (5'-T TACGTTATTATTGATGGTG, targeting GenBank Accession number NC_009089.1); the predicted T_m for *tcdB* is 25°C higher than for *tcsL*. To detect the internal control amplicon, a *nuc* capture probe (5'-GACAAAG GTCAAAGAACTGA, targeting GenBank accession number CP000730) was also synthesized.

Bacterial strains, genomic DNA, and clinical samples. Bacterial strains and genomic DNA were purchased from the ATCC (American Type Culture Collection, Manassas, VA) or CCUG (Culture Collection, University of Göteborg, Göteborg, Sweden). DNA was resuspended in water and quantitated by absorption at 260 nm. Samples were cultured anaerobically on tryptic soy agar plates supplemented with 5% defibrinated sheep's blood (Becton, Dickinson, Franklin Lakes, NJ) or in reinforced clostridial medium (Becton, Dickinson) at 37°C. Stool samples were collected and deidentified at clinical sites (Medical College of Wisconsin, Milwaukee, WI, and McLendon Clinical Laboratories, University of North Carolina, Chapel Hill, NC). Upon receipt at Great Basin Scientific, samples were thawed, and multiple swabs were taken and stored at -70°C. All specimens were collected under institutional review board-approved protocols.

Assay. Swabs taken from unformed stool samples were vortexed in extraction buffer ([EB] 750 μ l of phosphate-buffered saline [PBS]-0.01% Tween 20), filtered using a 3-ml syringe, and heated at 95°C for 5 min in 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 7.7 mM MgSO₄, 40 mM NaCl, 5 mg/ml bovine serum albumin (BSA), and 0.02% Tween 20 (Sigma Aldrich, St. Louis, MO). Twenty microliters was added to 20 μ l of 2 \times blocked-primer HDA (bpHDA) mix (20 mM Tris-HCl, pH 8.8, 40 mM NaCl, 0.8 mM each dCTP, dGTP, and dTTP, and 6.8 mM dATP), 10 ng/ μ l thermostable UvrD helicase (Tte-UvrD; BioHelix), 1.6 U/ μ l glutathione S-transferase (GST) polymerase (New England BioLabs, Ipswich, MA), 2 μ l of RN2 Master Mix (Great Basin Corporation), 4 ng/ μ l extreme thermostable single-stranded DNA binding protein ([ET SSB] New England BioLabs), EvaGreen (used at 0.4 \times ; 1 \times is the manufacturer-recommended final dilution; Biotium, Hayward, CA), 400 nM and 800 nM *tcdB* forward and reverse primer, respectively, and 200 nM and 400 nM *nuc* primers and incubated for 45 min at 65°C (LightCycler 480; Roche, Basel). Doubling time was calculated as described previously (33) from linear regression of a plot of $\ln(C. difficile \text{ cell input})$ versus crossing point (C_p), where doubling time is $\ln 2/\text{slope}$. Detection was performed as described

previously (22), and images were captured by a digital camera. If control features including detect, hybridization, and *nuc* each generated signal, the test was determined to be valid. If the *tcdB* feature generated visible signal, the test result was considered *tcdB* positive. If the test was valid and the *tcdB* feature did not signal, the result was *tcdB* negative.

Automation. A *C. difficile* ToxB assay was automated using an analyzer and disposable cartridge (Great Basin Corporation) that performs the DNA extraction, amplification, and detection steps within an enclosed system. A disposable cartridge is manufactured by injection molding, and channels and fluid chambers are formed by adhesion of a clear plastic to the cartridge. A 7-mm² silicon chip with capture probes is bonded within a detection chamber, blister packs that store liquid reagents are attached, and lyophilized HDA reagents are added. To perform a test, the operator swabs, vortexes the swab in EB, filters, and delivers 180 μ l into the cartridge. After the sample port is closed, the cartridge is inserted into the analyzer, sample information is entered, and the test is initiated using a graphical user interface. A lance pierces the blister pack containing extraction buffer, and a plunger compresses the blister, expelling liquid through a mesofluidic (0.5-mm² cross-sectional area) channel into the extraction chamber. Optical sensors that detect fluid movement trigger plunger motor and temperature control actions. Valves, controlled by two-position linear actuator motors, are closed to isolate the chamber. Mixing is accomplished via a magnetic stir bar, and the sample is heated via direct contact with a heater. A second dilution is performed in the downstream control chamber, again with mixing, and the amplification chamber is filled, rehydrating lyophilized HDA reagents. For isothermal DNA amplification, this chamber is fluidically isolated and maintained at 65 \pm 2°C by direct contact with a heat source. For detection, the amplified sample is diluted with hybridization buffer and introduced into a chamber where a 7-mm² silicon chip is affixed. As for prior steps, fluidic movements and heater control perform the hybridization, washing, and signal development steps. The resulting eye-visible features are captured by a digital camera. Processing and filtering techniques minimize background and maintain the required signal-to-noise level. Multiple custom algorithms query pixel intensity and intensity gradient directionality to determine the presence or absence of a signal on each array feature. Once the optical reader software has determined the presence or absence of signal on each array feature, a call logic tree is used to determine the assay result, which is reported automatically.

Clinical samples were tested with a BD GeneOhm *C. difficile* PCR assay as the reference method, performed at a clinical site according to the manufacturer's recommendations (Becton, Dickinson). In parallel, the sample was deidentified, blinded, and tested in singlet by the automated *C. difficile* ToxB assay. Each sample was from a different patient. The lone discrepant result was from a heavily mucoid sample. Upon homogenization with a wooden spatula and repeat testing, the sample was positive for *C. difficile* ToxB. This sample was therefore resolved as positive and scored as false negative.

To calculate the limit of detection (LoD), logistic regression was used to fit a plot of CFU input versus the observed detection counts, and inverse prediction was used to find the predicted CFU value with a 95% probability of detection.

RESULTS

Isothermal *tcdB* amplification using blocked primers. To amplify and detect the *tcdB* gene of toxigenic *C. difficile*, the assay

FIG 1 Assay scheme. (A) Cell lysis releases genomic DNA, which is unwound by a helicase. Primers bind and are extended by a DNA polymerase. After the initial unwinding and primer extension, exponential-phase amplification proceeds continuously at 65°C via helicase unwinding, primer binding, and polymerase extension. As the reaction nears completion, the nonbiotinylated primer is depleted, and the HDA enters an asymmetric phase in which the biotinylated primer produces single-stranded product. (B) Detection. Biotinylated amplified DNA is hybridized to DNA capture probes. A horseradish peroxidase (HRP)-conjugated antibody (Ab) binds to biotin, and TMB (3,3',5,5'-tetramethylbenzidine) oxidation results in product precipitation, producing a visible feature. (C) Imaging of chip patterns for positive (+) and negative (-) *tcdB* results. IC, internal control; HC hybridization control; DC, detection control; F, chip orientation feature; Tox, *tcdB*.

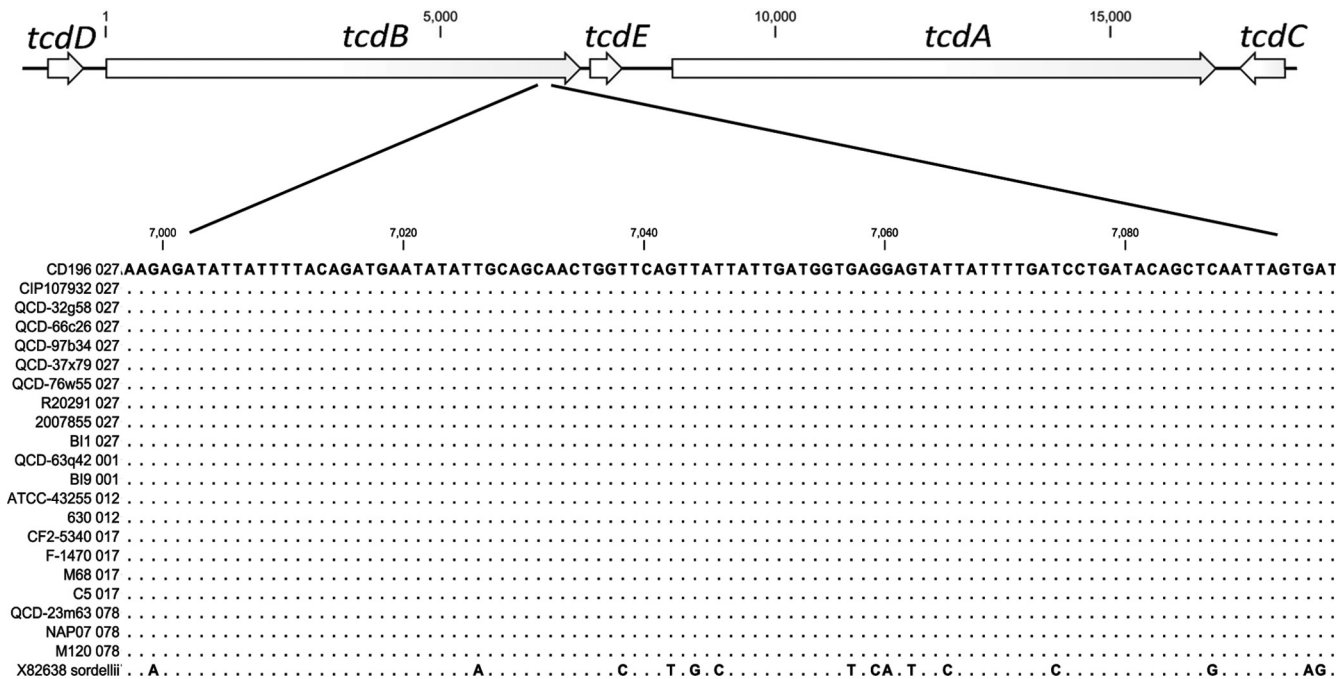


FIG 2 PaLoc and *tcdB* alignment. The 20-kb pathogenicity locus with genes and direction of transcription (arrows) is expanded in the targeted *tcdB* region. Sequences of 22 PaLocs, identified on the left of the figure, are shown. Numbering corresponds to *tcdB* sequence, where 1 is the start codon. Dots, invariant nucleotides. The bottom sequence represents *C. sordellii tcdB*.

(Fig. 1) utilizes helicase-dependent amplification (1) to generate an amplicon which is then hybridized to capture probes, finally generating a visible array pattern. Amplification primers were identified that bind to a 3' *tcdB* region that is completely conserved in sequence across 22 GenBank PaLoc sequences (Fig. 2). This region is downstream of a stretch of variable sequence within *tcdB* (31), in a region where PaLoc sequence variations and insertions/deletions are unknown. An HDA modification termed blocked-primer HDA (bpHDA) was adopted. In bpHDA, DNA polymerase cannot extend primers until an elevated temperature is reached and the primer is deblocked by a thermostable RNase, effectively creating a hot-start condition (Fig. 3A). Standard HDA and bpHDA modes were compared using a *tcdB* primer set in either blocked or unblocked form. After amplifying 100 copies of input *C. difficile* genomic DNA, products were analyzed by polyacrylamide gel electrophoresis (Fig. 3B), revealing a high yield of a bpHDA product with the expected mobility. In contrast, standard HDA showed a mixture of primer artifact and expected product at 100 copies of input DNA, with poor reproducibility between replicates.

Amplification rate. To examine bpHDA rate and sensitivity, genomic DNA was serially diluted, and the amplification time (C_p) for each template concentration was determined in LightCycler studies. As observed in Fig. 4, amplification doubling time was 28 s. The bpHDA reaction amplified a single copy of genomic DNA with a C_p of 17 min.

Assay readout and internal control. An internal control was integrated that amplifies and detects the *S. aureus nuc* gene, which is then detected by a *nuc*-specific capture probe. The *nuc* amplicon doubling time is slower than that of *tcdB*, resulting in a 7-min difference in amplification time (Fig. 5). To test whether the co-amplifying *nuc* gene alters assay sensitivity, 4,000 *S. aureus* CFU were tested using 0, 1, or 10 *C. difficile* CFU spiked into a pooled *C.*

difficile-negative stool sample. With 0 *C. difficile* input CFU, *nuc* amplified and produced a characteristic visual chip pattern. At a *C. difficile* input of 1 and 10 CFU, the *tcdB* amplicon efficiently amplified, displaying a distinct chip pattern (Fig. 5). This indicated that 1 CFU could displace amplification of the internal control under these conditions, while lack of target CFU resulted in internal control amplification and detection. Taken together, *tcdB* and process control features verify assay function and report toxigenic *C. difficile* status.

Automated assay: analytical sensitivity, specificity, and testing of clinical samples. An electromechanical instrument and dis-

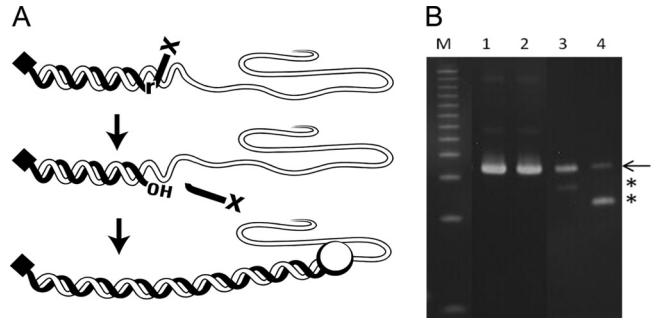


FIG 3 Blocked-primer HDA. (A) A blocked primer bound to the target sequence cannot be extended by DNA polymerase (circle) due to a 3' blocking group (X, a 3-carbon spacer). At elevated temperatures, optimally 60 to 70°C, *Pyrococcus abyssi* RNase H2 is activated, and a ribonucleotide (r) is cleaved, liberating a 3' hydroxyl group (OH) and allowing primer extension. bpHDA operates at the primer binding step of the Fig. 1 HDA scheme. (B) Gel separation of replicate bpHDA (lanes 1 and 2) and standard HDA (lanes 3 and 4) products, stained with SYBR Gold dye. Lane M, 25-bp DNA ladder. Arrow, mobility of desired amplification product; asterisk, amplification artifact.

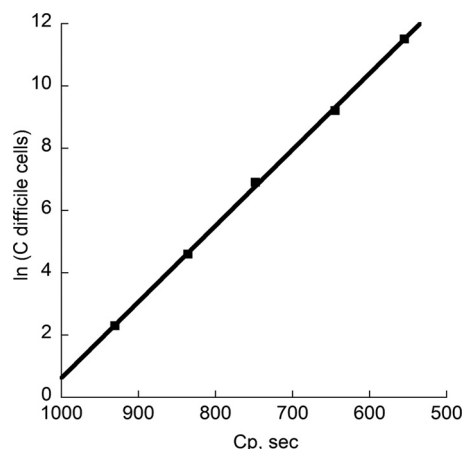


FIG 4 bpHDA amplification rate. Various cell input amounts were amplified by bpHDA, and $\ln(C. difficile \text{ cell count})$ was plotted against C_p values. The line represents best-fit linear regression.

posable cartridge were developed, and the automated assay was optimized to function equivalently to the manual assay in incubation times and temperatures. The disposable cartridge contains a port for sample introduction, control chambers for heating and mixing to extract DNA, an amplification chamber, and a detection chamber that houses the silicon chip (Fig. 6). After the filtered sample is loaded, the assay is initiated using a graphical user interface. After 90 min, the *C. difficile* ToxB test result is returned. Analytical sensitivity was addressed using dilutions of cultured *C. difficile* spiked into a pooled negative stool sample; at 20 CFU input, 20/20 tests were positive. At 10 CFU input, 10/11 tests were positive, and at 4 CFU input, 6/19 tests were positive. Inverse prediction based on a logistic regression model fit to these data indicated that the automated assay detection limit is 10 CFU input to an amplification reaction (95% probability of detection). We then determined assay reactivity toward several *C. difficile* strains as well as toxigenic *C. sordellii* and nonclostridial species that can be present in stool samples. Each organism was spiked into a negative stool sample, and subsequent chip readouts indicated that all toxigenic *C. difficile* strains were detected, while toxigenic *C. sordellii*, nontoxigenic *C. difficile*, and nonclostridial species tested negative (Table 1). Finally, to determine the ability to detect toxigenic *C. difficile* in clinical samples, 130 samples were tested alongside an FDA-approved PCR test. Discrepancies were resolved by toxigenic culture. Of these samples, one false negative was detected among the 32 positive samples, and no false positives were observed, yielding 97% sensitivity (95% confidence interval [CI], 82 to 99%) and 100% specificity (95% CI, 95 to 100%). These initial experiments demonstrated automated assay function, paving the way for larger-scale prospective clinical studies.

DISCUSSION

To combine the advantages of molecular testing (sensitivity) and immunoassays (low cost), we developed an assay for toxigenic *C. difficile* that couples isothermal DNA amplification to array-based hybridization. In lieu of monitoring nucleic acid amplification in real time, this approach permits inexpensive detection, requiring only a digital image instead of fluorophore-based detection with accompanying sophisticated optics and algorithms. Multiplexing is accomplished at two levels: at the amplification step and via

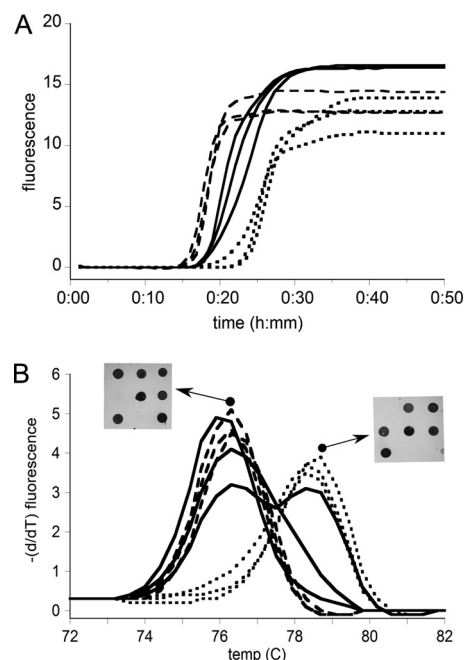


FIG 5 Internal control. Cells were spiked into a pooled negative fecal sample. (A) Amplification curves, in triplicate, of 4,000 CFU of the internal control cells (IC; dotted lines) or 4,000 CFU of the internal control cells plus 1 (solid) or 10 (dashed) CFU of *C. difficile*. (B) Melting analysis of the nine reactions shown in panel A. Amplified material from representative reaction mixtures containing the internal control cells only or the internal control cells plus *C. difficile* was detected on chips as indicated. The chip legend is shown in Fig. 1C.

hybridization to capture probes immobilized on the array. These methods were sufficient for detection of fewer than 10 *C. difficile* CFU in the context of a fecal sample. The ability of HDA to amplify crude fecal samples is also seen with other crude samples, for example, blood culture (22). Straightforward filtration and automated dilution produce a simple test in which a swab sample is filtered and transferred into the cartridge to initiate testing.

To discriminate toxigenic from nontoxigenic *C. difficile* bacteria, we identified a PaLoc region that is completely conserved in sequence among known *C. difficile* isolates. Repetitive elements within *tcdA* and *tcdC* contain deletions that reflect the plastic nature of the *C. difficile* pathogenicity locus. Included within these variants are a nontoxigenic strain that bears a remnant 3' fragment of *tcdA* (25); in addition, new *tcdA* variants are being described (15). There are no known clinical isolates that are TcdA⁺ TcdB⁻, whereas virulent TcdA⁻ TcdB⁺ strains are known. Taken together, these observations indicate that targeting *tcdA* or *tcdC* alone could lead to false-positive or false-negative test results unless care is taken to amplify a genetically stable region. Using an alignment of 22 PaLoc genes, including representatives from diverse *C. difficile* clades (11, 18), primers were designed that amplify a 78-bp target sequence. The *tcdB* gene is not subject to any known deletions, and therefore the assay result invariably correlates with toxigenic status.

In comparison to the PCR, HDA uses a helicase to separate DNA strands instead of thermal denaturation. The bpHDA enhancement presented here suppresses amplification artifacts by effectively creating a hot-start amplification condition analogous to hot-start PCR approaches. In bpHDA primers are blocked by a

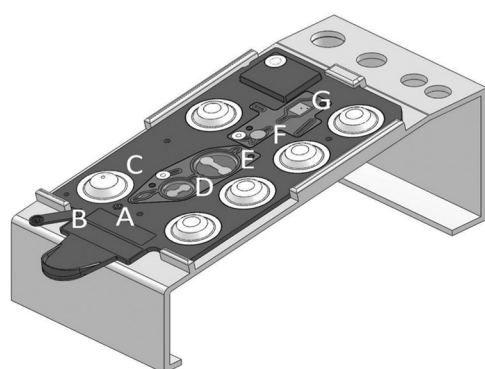


FIG 6 Cartridge and analyzer. The injection-molded cartridge, shown resting on a sample loading jig, contains a sample port (A) with hinged lid (B), blister packs containing liquid reagents (C), chambers for extraction (D) and dilution (E) containing magnetic stir bars, an amplification chamber (F), and a detection chamber with a chip (G). After sample loading, the cartridge is sealed using the hinged lid and placed into the analyzer as shown. The analyzer moves fluids through channels connecting the blister packs and control chambers, mixing and heating where needed, and finally capturing a chip image which is processed to return assay results.

3' group that prevents DNA polymerase extension. RNase cleavage at an upstream ribonucleotide reveals a 3' hydroxyl, and the primer can now be extended. Key to the process is a thermostable RNase that is inactive at temperatures below 50°C, as demonstrated in work that comprehensively details use of blocked primers in the PCR (12). Multiplexed HDA without a hot-start approach has been nicely demonstrated in a diagnostic test for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (13), using optimal primer concentrations of 40 to 150 nM but relatively long amplification times of 90 to 120 min. When reaction rates are measured in a real-time HDA mode analogous to real-time PCR,

the bpHDA enhancement amplified a single genomic DNA copy in 17 min. The corresponding bpHDA doubling time of 28 s exceeds the theoretical capacity of commercial PCR instruments, which are limited by instrument thermal cycling rates. Instead, the HDA is rate-limited only by enzymatic processes.

A limitation to HDA is that amplicon lengths of <120 bp are generally required. UvrD helicase, such as that used here, has low processivity of ~40 bp, a possible explanation for the improved efficiency of shorter bpHDA amplicons. Increasing UvrD apparent processivity could increase the practical amplicon length of HDA.

PCR instruments used for moderately complex molecular diagnoses use microfluidics that require high manufacturing precision, precise temperature control for thermal cycling, and sophisticated optics for fluorescence detection. These requirements constrain instrument and test costs. In contrast, the analyzer/cartridge described here provides meso-scale fluidic movement, isothermal amplification, and eye-visible detection. Mesofluidic channels enable injection molding of a single plastic part. The isothermal DNA amplification is tolerant to variations of at least $\pm 2^\circ\text{C}$, obviating the need for precise and rapid temperature changes that occur, perforce, in the PCR. By use of large visible features, the detection system can employ a digital camera rather than an expensive charge-coupled-device (CCD) imager. Taken together, mesofluidic design, isothermal DNA amplification, and eye-visible detection enable use of off-the-shelf components for analyzer construction, driving down instrument complexity and cost while maintaining ease of use. A limitation to the current automated test is the turnaround time of 90 min after test initiation, while the manual assay is performed in 60 min. The additional time is taken up by motor movements and mechanical calibrations; these factors have since been minimized to produce a 75-min test.

Several assays for *C. difficile* DNA amplification and detection have been reported. Among these, an HDA method detected 20 copies of purified genomic DNA; because a manual DNA extraction was required and because sensitivity for *C. difficile* CFU was

TABLE 1 Assay specificity^a

Organism	Strain	Toxin production	<i>C. difficile</i> ToxB result ^b
<i>Clostridium difficile</i>	ATCC 43255	A and B	Pos
	ATCC 43600	A and B	Pos
	ATCC 43599	A and B	Pos
	ATCC 17857	A and B	Pos
	BAA-1805	A and B	Pos
	BAA-1382	A and B	Pos
	CCUG 20309	B only	Pos
	ATCC 43598	B only	Pos
	ATCC 43593		Neg
<i>Clostridium sordellii</i>	ATCC 9714	<i>tcsL</i>	Neg
<i>Campylobacter jejuni</i>	ATCC 33560		Neg
<i>Citrobacter freundii</i>	ATCC 8090		Neg
<i>Enterococcus faecalis</i>	ATCC 29212		Neg
<i>Escherichia coli</i>	ATCC 4157		Neg
<i>Listeria monocytogenes</i>	ATCC 6896		Neg
<i>Proteus vulgaris</i>	ATCC 6896		Neg
<i>Pseudomonas aeruginosa</i>	ATCC 10145		Neg
<i>Salmonella enterica</i>	ATCC 13311		Neg
<i>Shigella flexneri</i>	ATCC 25929		Neg
<i>Staphylococcus epidermidis</i>	ATCC 12228		Neg

^a Organisms were cultured, spiked into a pooled negative fecal sample, and tested.

^b Pos, positive; Neg, negative.

not reported, it is difficult to compare the two assays directly (10). The automated *C. difficile* ToxB assay described here, employing bpHDA and a minimal sample preparation procedure, has an LoD of 10 CFU input (at 95% detection confidence), while the manual assay could reliably detect an input as low as 1 CFU. Thus, the bpHDA method is comparable in sensitivity to other PaLoc amplification methods (7, 10, 20, 23, 24), many of which require DNA purification prior to amplification. These experiments demonstrated that the manually developed assay was successfully automated. Initial assessment using clinical samples suggests that this is an accurate test, and large studies are now required to establish clinical sensitivity and specificity.

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